PRO-2-PAM: THE FIRST THERAPEUTIC DRUG FOR REACTIVATION OF ORGANO-PHOSPHATE-INHIBITED CENTRAL (BRAIN) AND PERIPHERAL CHOLINESTERASES

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ABSTRACT

Due to the documented use of organophosphorus (OP) chemical agents in warfare and by terrorists around the globe, Federal, State, and local authorities need novel therapeutics to overcome their deleterious effects. OPs inhibit cholinesterases (ChE), leading to accumulation of the neurotransmitter acetylcholine (ACh). Potentially lethal effects begin with secretion, muscle fasciculation, and paralysis in the peripheral nervous system (PNS). Central nervous system (CNS) perturbations include epileptic seizures leading to neuronal damage and long-term structural changes. Therapy for OP exposure is a combination of atropine sulfate to block the overload of cholinergic (muscarinic) receptors, the FDA approved cholinesterase reactivator (oxime) pralidoxime chloride (2-PAM), and a benzodiazepine (diazepam) anticonvulsant to ameliorate seizures. However, current therapies for acute pesticide or organophosphate poisoning do not provide treatment for centrally inhibited cholinesterases because quaternary nitrogen charged oximes, including 2-PAM, do not cross the blood brain barrier.

Since there is no direct CNS action of 2-PAM as a countermeasure to OP exposure, there is a critical need for new therapeutics to improve patient survival and limit the profound CNS effects. Therefore, to reactivate CNS acetylcholinesterase (AChE), we first synthesized and then evaluated the ability of pro-2-PAM, a derivative of 2-PAM that is lipid permeable based on its logP, to enter the brain and prevent seizures after exposure to an OP agent in guinea pigs. The protective effects of the pro-oxime after OP exposure were documented and correlated using a) surgically implanted radiotelemetry probes that recorded electrocardiogram (ECG), electroencephalogram (EEG), body temperature, and physical activity, b) histopathology analysis of brain, and c) cholinesterase activities in the PNS and CNS. The PNS oxime 2-PAM was ineffective at reducing seizures/*status epilepticus* (*SE*) in diisopropylfluorophosphate (DFP)-exposed animals. In contrast, pro-2-PAM significantly suppressed and then eliminated seizure activity. Consistent with this observation, distinct regional areas of the brains showed significantly higher AChE activity at 1.5 h after OP exposure in pro-2-PAM treated animals compared to 2-PAM treated guinea pigs. However, blood and diaphragm showed similar AChE activities for animals treated with either of the two oximes, since 2-PAM is a PNS active oxime and pro-2-PAM also retains this capability. In conclusion, our results show that pro-2-PAM is able to cross the blood-brain barrier (BBB), is rapidly metabolized inside the brain to 2-PAM, and protects against OP-induced seizures (*SE*) through restoration of brain AChE activity.

The product of this work will be the first non-invasive means of administering a therapeutic for the deleterious effects of OP poisoning in the CNS by reactivating CNS ChE. This pro-oxime fulfills the requirement for rapid and reliable PNS and CNS cholinesterase reactivation in both military and civilian populations.

1. INTRODUCTION

Organophosphorus chemical warfare nerve agents (CWNA) inhibit the catalytic site of AChE and butyrylcholinesterase (BChE), leading to a build up of ACh in the CNS and PNS that disrupts cholinergic neurotransmission (Taylor, 1990). If severe enough and left untreated, AChE inhibition ultimately results in a cholinergic crisis and death. In survivors, prolonged and repeated *status epilepticus* seizures result in severe neuropathology and neurobehavioral abnormalities (Carpentier et al, 2000). Currently, there are no direct treatments for acute OP poisoning of CNS inhibited ChE (Marrs et al, 2006). Therapy for OP exposure includes combined administration of a ChE reactivator (the PNS active oxime 2-PAM), a muscarinic receptor antagonist (CNS active atropine), and an anticonvulsant (CNS active diazepam) (Newmark, 2004a). Atropine and 2-PAM therapy alone is ineffective for OP-induced seizures once they are established, but these antidotes in combination with the benzodiazepine anticonvulsant decrease seizures associated with nerve

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agent exposure. However, diazepam has limited bioavailability when administered intramuscularly by the use of an auto-injector, and its efficacy is also reduced after prolonged seizure activity. This drug combination significantly increases survival, but does not treat the origin of seizure in the brain – inhibition of CNS ChEs. Furthermore, drugs that suppress the CNS activity, such as diazepam, have the potential for over-sedation and respiratory depression, especially where patients may be suffering from hypoxia due to diaphragm paralysis from CWNA.

CNS seizure/*SE* is an outcome of OP exposure because 2-PAM does not penetrate the BBB, and thus cannot reactivate OP-inhibited CNS ChEs. Since there is no catabolism of the neurotransmitter ACh, excessive receptor stimulation by ACh is only partially overcome by the antimuscarinic and anticonvulsant drugs. This is followed by a cascade of excitatory amino acid release resulting in neuronal damage associated with *SE*. The repeated prolonged seizure characteristic of *SE* may lead to neuronal death and permanent brain damage (Lowenstein, 1999).

It is noteworthy that the OP G-nerve agents, including tabun (GA), sarin (GB), soman (GD), and VX readily penetrate through the BBB. Log P (octanol:water partition coefficients) values reflect lipid solubility of a compound and predict its distribution in the body. Nerve agents exhibit high coefficients which predict absorption through the skin and distribution of OP compounds to the CNS. Log P values were correlated with the onset of local fasciculation and toxicity in guinea pigs, reflecting dissemination throughout the animal (Czerwinski et al, 2006).

AChE inhibitors are used as both therapeutic treatments for glaucoma, myasthenia gravis, Alzheimer's disease (as a CNS therapeutic) or atropine poisoning, and on the other hand, in sinister ways including attacks by terrorists and military warfare to kill humans (Martin and Lobert, 2003). In 1940, the German Army Weapons Office ordered the mass manufacture of sarin. Evidence of the use of sarin in warfare by Iraq against its Kurdish population and in its military conflict against Iran was documented by U.N. inspectors after the 1988 war (Newmark, 2004b). Sarin was used against civilians in the Tokyo subway terrorist event in 1995. Notably, this event resulted in documented long-term neuronal sequelae characterized by CNS-associated behavioral and cognitive alterations despite conventional oxime therapy with 2-PAM (Miyaki et al, 2005; Yamasue et al, 2007). This incident resulted in twelve deaths and more than 1,000 injured with still undetermined CNS consequences.

Oximes regenerate OP-inhibited AChE through nucleophilic attack of the oxime's oxygen on the electrophilic phosphorus of the OP bound to the serine in the active site of the ChE (Taylor, 1990). Pesticides and CWNA undergo a deacylation process known as aging, during which the enzyme becomes refractory to reactivation by oximes. Aging times vary depending on the OP (Clement, 1982). GD ages AChE in about 2 minutes, and therefore requires oxime to be on board as soon as possible, while GB ages AChE in 3–4 hours, so oximes can be efficacious later. Due to their chemical structure, positively charged, hydrophilic small molecule oximes - 2-PAM, obidoxime, HI-6, and MMB-4 - do not penetrate the BBB and therefore cannot reactivate OP-inhibited ChEs in the brain (Bajgar et al, 2007). Since there is no direct action on the CNS by this treatment as a countermeasure to OP exposure, there is a critical need for new therapeutics to improve survival and limit CNS effects after CWNA exposure.

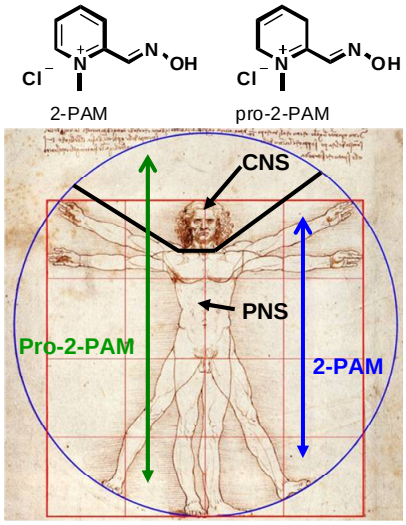

Fig. 1. Top: Structures of oximes: 2-PAM (left) and pro-2-PAM (right). *In vivo*, pro-2-PAM is converted to 2-PAM. Bottom: Pro-2-PAM but not 2-PAM penetrates the PNS *and* CNS.

To overcome the lack of BBB penetration by these oximes, we investigated pro-2-PAM, the pro-drug of 2-PAM (Fig. 1), which has a dihydropyridyl versus a pyridyl ring, respectively. This pro-drug permits stealth entrance into the brain due to its increased lipophilicity as shown by its calculated positive log P compared to 2-PAM, 0.2738 and -3.666, respectively. Once in the CNS, pro-2-PAM is rapidly converted back to its parent compound and therapeutic oxime 2-PAM by non-specific oxidases (Bodor, 1976) or flavins in blood or cerebral spinal fluid (unpublished observations). It then reactivates CNS (as well as PNS) inhibited ChEs. The specific hypothesis we tested was that a BBB-penetrating oxime, pro-2-PAM, can reactivate central AChE and reduce the sequelae of a CNS cholinergic crisis, especially neuropathy, in response to OP chemical agent exposure.

2. METHODS

2.1 Synthesis of pro-2-PAM (Fig. 1). We synthesized the pro-drug, pro-2-PAM, as previously described (Bodor, 1976). However, the final step, the E1 elimination of hydrogen cyanide, took significantly longer than reported, but storing the reaction mixture at 4^{o}C overnight resulted in precipitation of additional pro-2-PAM product. The drug was fully characterized by ^{1}H and ^{13}C NMR, ele-

mental analysis, mass spectrometry, reverse phase HPLC, and its bioactivity by AChE reactivation assay after oxidation to 2-PAM *in vitro*. Pro-2-PAM is stored as a readily water soluble powder, similar to the oxime HI-6.

2.2 WRAIR cholinesterase assay (Feaster et al, 2004)**.** The WRAIR Assay was performed in 96-well microtiter plates; the final concentrations of substrates were 1 mM each of acetylthiocholine, propionylthiocholine, butyrylthiocholine iodides, and 0.2 mM 4,4' dithiodipyridine, the indicator for the hydrolyzed thiocholine, and UV absorbance measured at 324 nm, which avoids the hemoglobin interference observed with Ellman's reagent (Haigh et al, 2008). Guinea pig blood was collected with heparin and tissues were frozen at -80°C. To perform the ChE assays, a small aliquot of blood, typically 10 µL, was diluted 20-fold in distilled water. Tissues, in a Covaris (Woburn, MA) CryoPrep bag, were crushed 3 times at high setting, weighed and added (1:7 w/v) to tissue protein extraction reagent (TPER, pH 7.6, Pierce Chemicals, Rockford, IL). Samples were homogenized (on ice) using a ground glass homogenizer until the tissue was completely emulsified. The homogenate was centrifuged for 10 min at 15,000 x *g* at 4°C and the supernatants assayed for ChE activity in triplicate (final volume of 300 µL using 50 mM sodium phosphate buffer, pH 8.0). A four-minute kinetic assay was performed at 25°C using a Molecular Devices SpectraMax Plus^{384} microtiter spectrophotometer (Sunnyvale, CA). Data were subjected to linear least squares analysis from which the activities of AChE and BChE (U/mL) were calculated using SoftMax v5.2 and an Excel spreadsheet. A 2-tail t-test was used with a p-value of ≤ 0.05 to indicate significance.

2.3 Animal surgery. Two month old adult male guinea pigs were fasted for several hours, anesthetized (2-5% isoflurane, oxygen 1.5 L/min), shaved on the head and back, and their heads placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). The radiotelemetry system consisted of 8 receivers and TL11M2-F40-EET bipotential radiotelemetry probes (DSI, St. Paul, MN) turned on and off by hand with a magnet. Probes were reused and sterilized using 4% glutaraldehyde and handled as instructed by the manufacturer. Briefly, the surgery proceeded as follows: radiotelemetry probes were surgically implanted under the back skin, with wire leads fixed to the skull, chest muscle, and abdominal muscle to record brain activity (EEG), heart rate (ECG), and body temperature, respectively (Tetz et al, 2006) (Fig. 2, left). Cyano-acrylate glue was used to keep the skull electrodes in place. Incisions were sutured using Ethicon sutures (Piscataway, NJ) and covered with TissueMend glue (Webster's Veterinary Supply, Sterling, MA). The guinea pigs were housed individually in microisolator cages with a 12 hour light/dark cycle. Food and water were available *ad libitum*, and a one week stabilization period preceded surgery and experimentation.

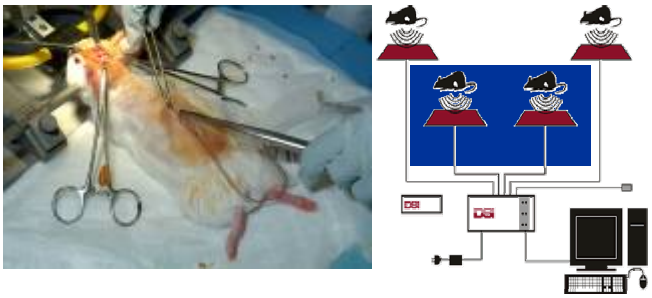

Fig. 2. Left: Surgical probe implantation. Guinea pig's nose is in an isofluorane delivery cone. EEG leads tunneled to screws in skull, ECG/body temperature leads in chest/abdominal. Probe is sutured in the back, s.c. Right: Schematic of 24 h radiotelemetry recorder. The "red" pads receive the probe's radio signal, permitting untethered animal movement, which is processed, stored, and visualized on the computer.

2.4 Radiotelemetry. On the day of the experiment, the standard military exposure paradigm was used and only the oxime delivery time was modified (Newmark, 2004a) as follows: guinea pigs were pretreated (i.p.) with the FDA approved prophylactic drug pyridostigmine bromide (PB) at 0.026 mg/kg. PB is a reversible inhibitor of AChE activity, but does not cross the BBB and therefore does not sequester CNS ChEs. After 20 minutes, the animals were injected (s.c.) with DFP (8 mg/kg) followed 1 min later by atropine methyl bromide (i.m., 2 mg/kg). At various times post-OP exposure, equivalent doses of 2-PAM or pro-2-PAM were injected i.m. (1.5 auto-injector, 13 mg/kg) to approximate use of the Mark I nerve agent antidote kit provided to military personnel. The EEG, ECG, body temperature, and activity of the animals were continuously monitored and telemetry recorded for 24 h (Fig. 2, right). Guinea pigs were euthanized after 24 h (in some studies 1.5 h) by injecting 75 mg/kg pentobarbital followed by terminal cardiac puncture exsanguination. Brain, blood, and diaphragm tissues were frozen on dry-ice for ChE assays. Some whole brains, taken from heparinized saline perfused animals, were thawed and dissected into eight distinct brain regions for AChE assay: frontal cortex, rear cortex, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, and brain stem.

2.5. Histopathology. Twenty four hours post-exposure, guinea pigs were euthanized as above, the brain removed, and forebrain taken for ChE activity assay. The remainder of the brain was subjected to immersion fixation, for at least several weeks, in 4% formaldehyde (stabilized with 0.5% methanol). Next, the formaldehyde-preserved guinea pig brains were transverse sectioned using a rodent brain matrix (model: RMB-5000C; ASI Instruments, Inc., Warren, MI). Two sequential transverse sections, "A" and "B", of 2 mm thickness were cut from each brain, using microtome blades hand dropped into the matrix. Section "A" was cut starting at the nose of hippocampus and section "B" was cut starting near the back of the hippocampus, adjacent to the midbrain. Both sections were proc-

essed into microscope slides containing paraffin embedded 6 µm transverse sections (microtome cut) stained with hematoxylin and eosin (H&E) or fluoro-jade in duplicate (FD Neurotechnologies, Inc; Ellicott City, MD). H&E stain is reactive towards membrane lipids and proteins, and highlights the general structural morphology of all cells. In contrast, fluoro-jade stain penetrates only leaky membranes and thus highlights dead cells. Prepared slides were examined at 40x magnification under an Olympus axial light microscope equipped with an image capture camera (Olympus Provis AX80/DP70; Olympus, Center Valley, PA). Standard bright field and fluorescence (FITC filter) illuminations were used on the H&E and fluoro-jade stained slides, respectively. The middle lobe of the piriform cortex, a distinct brain region known to be sensitive to OP nerve-agent induced damage and a site of seizure initiation/propagation (Carpentier et al, 2000), was examined in the section "A" slides. Likewise, in the section "B" slides, the lower-outside pyramidal layer of the hippocampus (CA1-CA2 region) was chosen for examination. Photographic images were captured of neurons and granular cells comprising the selected regional zones in both sections.

3. RESULTS

3.1 Radiotelemetry: pro-2-PAM but not 2-PAM abrogates DFP-induced *status epilepticus*. We compared the two oximes in guinea pigs, the model for OP poisoning because its repertoire of OP detoxifying enzymes matches the human enzyme complement. Control, DFP alone, or DFP followed by the oximes 2-PAM or pro-2-PAM treated animals were continuously monitored for 24 h for brain activity (EEG, Fig. 3) and heart rate (ECG), body temperature, and physical activity (Fig. 4). These parameters allowed us to evaluate the protective effects of pro-2-PAM in comparison to 2-PAM after OP (DFP) exposure. The amount of oximes (2-PAM or pro-2-PAM) administered as a single dose was equivalent to 1.5 human auto-injector dose by body weight, which was found to be most efficacious, although we also evaluated doses of 1, 2, and 3 auto-injector equivalents (not shown). Control animals received PB, atropine, and saline instead of DFP and/or oximes.

Control guinea pigs displayed EEG tracing with only minor single spikes due to instrumentation noise (Fig. 3, Control). In contrast, guinea pigs exposed to DFP produced intense *SE* seizures that continued for the full 24 h, if they survived (Fig. 3, DFP). The oxime 2-PAM was ineffective at reducing seizures/*SE* in DFP-exposed animals (Fig. 3, 2-PAM), since the guinea pigs exhibited seizures for the full 24 h recording period. Note that 2-PAM was rapidly injected at 1 min post-DFP exposure. These data support reports that 2-PAM can not pass the

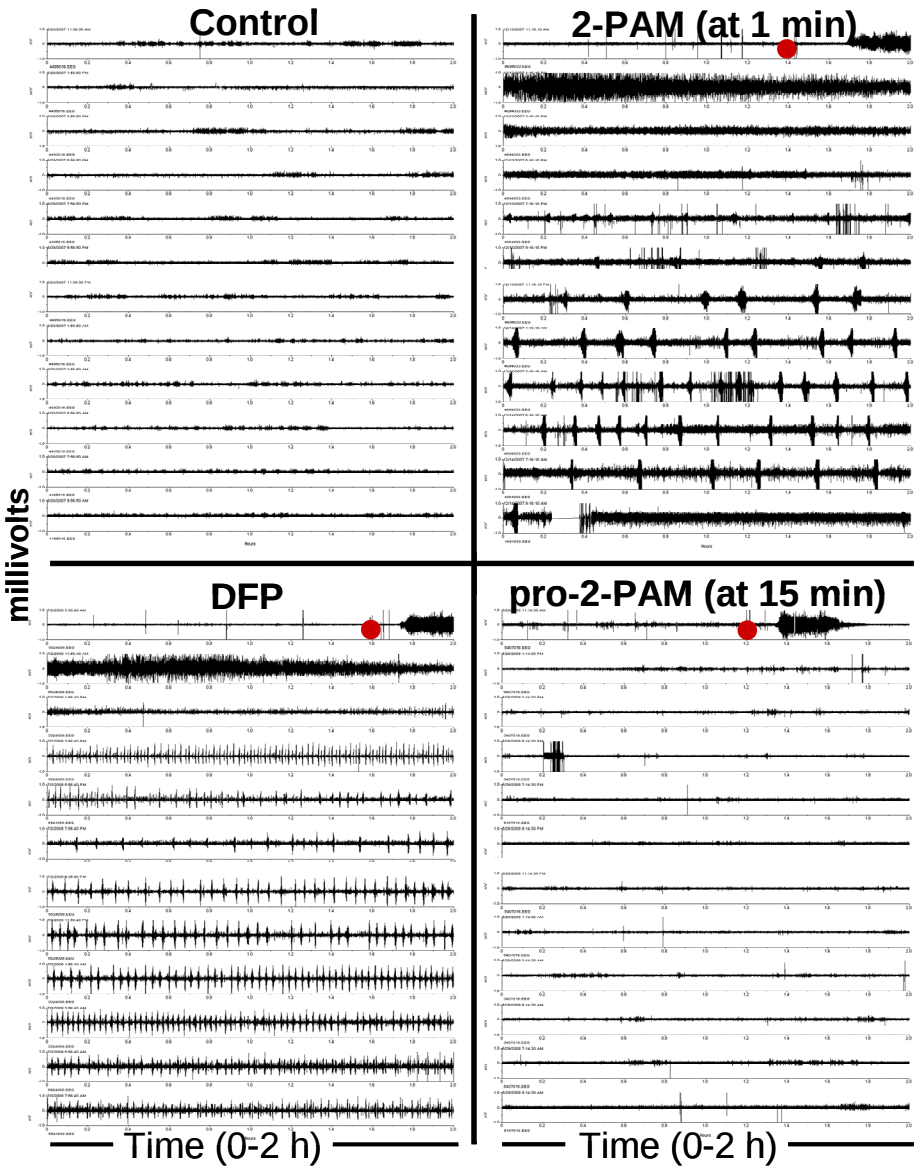

Fig. 3. Representative EEG traces for Control, DFP, DFP then 2-PAM, and DFP then pro-2-PAM treated guinea pigs. Animals received the standard military paradigm (see Methods, section 2.4), e.g., 2-PAM 1 min post-exposure *except* for pro-2-PAM treatment which was delayed by 15 min. This clearly demonstrates the efficacy of pro-2-PAM to alleviate *SE* at later times. Each line of EEG data represents 2 h of recording; a full 24 h are shown (12 lines) for each animal treatment. Red circle is time of DFP exposure.

BBB at therapeutically relevant doses (Bajgar et al, 2007; Kant et al, 2001). In notable contrast, pro-2-PAM prevented seizure activity in the standard military regimen of dosage and time, 1 min after OP exposure (not shown). Remarkably, pro-2-PAM abrogated seizure activity even when injected 15 min later (Fig. 3, pro-2-PAM). Clearly, pro-2-PAM - but not 2-PAM - may extend the time to treat OP-exposed soldiers for *SE*.

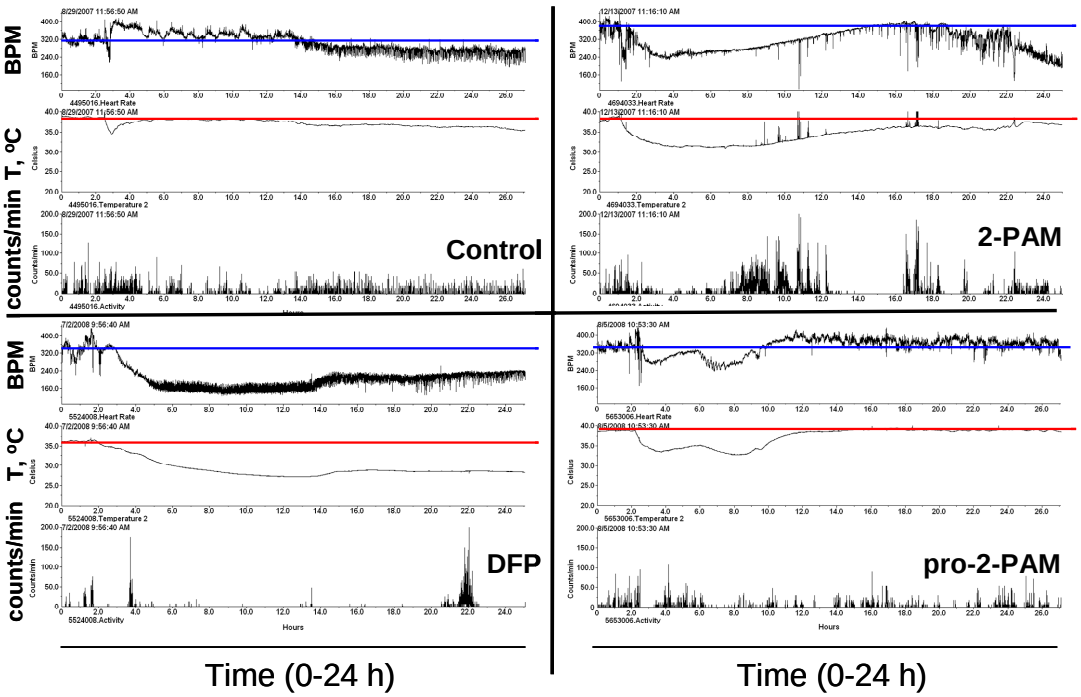

Fig. 4. 24 h radiotelemetry of heart rate (BPM, top), body temperature (T, °C, middle), and physical activity (counts/min, bottom) for control, DFP, DFP then 2-PAM, and DFP then pro-2-PAM treated guinea pigs. The horizontal bar denotes the initial heart rate (blue) and body temperature (red). Pro-2-PAM, and to a lesser extent 2-PAM, restored heart rate, body temperature, and physical activity depressed by DFP.

3.2. Radiotelemetry: pro-2-PAM restores body temperature and physical activity. Additional parameters of heart rate (BPM), body temperature (T, °C), and physical activity (counts/min) were recorded for 24 h by the radiotelemetry system (Fig. 4). The Control panel (Fig. 4) is representative of an animal's heart rate (top), body temperature (middle), and physical activity (bottom) after receiving only PB and atropine. DFP exposure results in prolonged hypothermia and bradycardia, and markedly decreased activity, all of which remained depressed for the full 24 h recording period (Fig. 4, DFP). As reported (Gordon, 1996), OP exposure causes hypothermia, bradycardia, and decreased activity due to fasciculation and fatigue. Treatment with 2-PAM partially modulated these responses (Fig. 4, 2-PAM). For instance, a long lag phase is observed before body temperature returns to normal. In distinct contrast to 2-PAM therapy, pro-2-PAM treatment abrogated DFP induced hypothermia and bradycardia and restored activity (Fig. 4, pro-2-PAM).

3.3 Cholinesterase assays of peripheral and central nervous system tissues. Consistent with the observations of protection of the CNS from *SE* by pro-2-PAM, but not 2-PAM, six of eight distinct regional areas of brain showed significantly higher ($p \leq 0.05$) AChE activity, at least 2-fold, at 1.5 h after DFP exposure in pro-2-PAM treated animals compared to 2-PAM treated animals (Fig. 5, pro-2-PAM and 2-PAM, red and black lines, respectively). Also consistent with the observation that PB does not penetrate the BBB (Kant, 2001), there was little inhibition of the eight specific brain regions by PB treatment

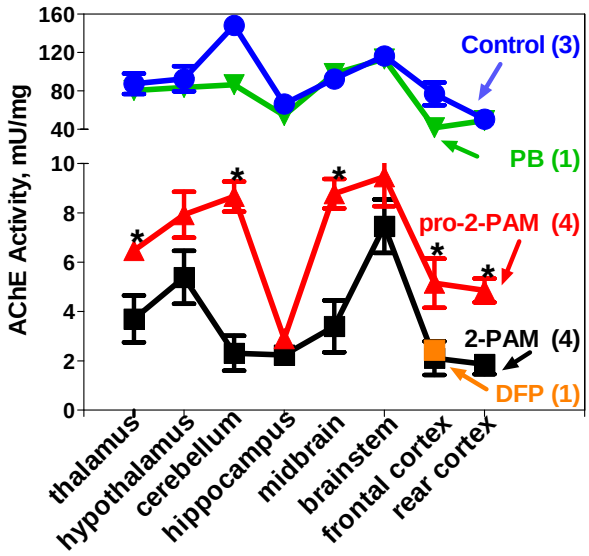

Fig. 5. AChE activity (mU/mg) in eight specific brain regions from guinea pigs 1.5 h after treatment with saline (Control, blue), PB (green), DFP only (orange), or animals treated with DFP followed by the oximes 2-PAM (black) or pro-2-PAM (red). The number of animals is in brackets. * indicates significant difference between pro-2-PAM and 2-PAM treatments; $p \leq 0.05$.

(Fig. 5, control and PB curves, blue and green, respectively). The data in Fig. 5 demonstrated that 2-PAM did not pass the BBB nor reactivate frontal cortex AChE

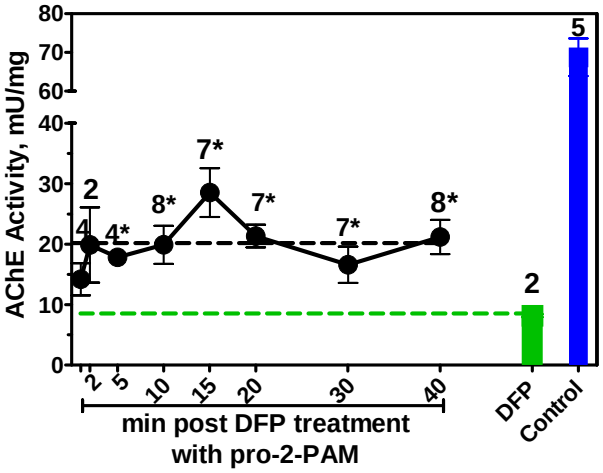

Fig. 6 Guinea pig brain (frontal cortex) AChE activity (mU/mg) at 24 h after treatment with pro-2-PAM at indicated times *after* DFP exposure. These data show that pro-2-PAM reactivated DFP-inhibited brain AChE when given up to 40 min post-OP exposure. Numbers above points are animals tested. Dashed lines are average AChE activity for pro-2-PAM treated animals (black) compared to DFP only animals (green). * indicates significant difference between pro-2-PAM treatment and DFP without oxime treated animals; $p \leq 0.05$.

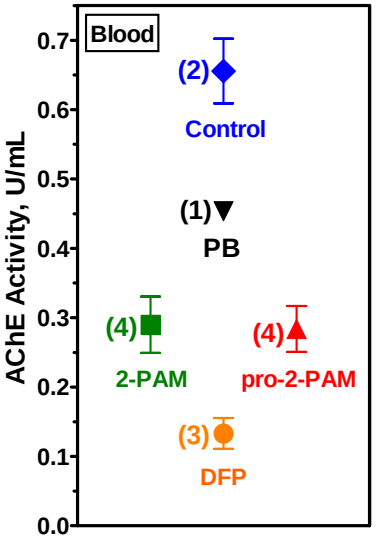

Fig. 7. Guinea pig blood AChE activity (U/mL) at 1.5 h post treatment. These data show 2-PAM and pro-2-PAM equivalence for peripheral reactivation of DFP-inhibited AChE. Numbers in brackets are animals tested.

since DFP (orange) and 2-PAM treated animal (black) showed the same inhibited AChE activity.

As described in section 3.1, pro-2-PAM was efficacious in stopping seizures when injected i.m. 15 min post-DFP exposure. Figure 6 shows the AChE activity of brain frontal cortex at 24 h with pro-2-PAM therapy between 1 and 40 min post-DFP exposure. There was greater than a 2-fold average increase in AChE activity (black dashed line) compared to DFP only treated animals (green dashed line) ($p \leq 0.05$). Thus, pro-2-PAM partially restored CNS AChE.

In the periphery, blood, which contains both red blood cell bound AChE and plasma BChE, has been used as a marker for OP inhibition and oxime reactivation (Thiermann et al, 2007). Similar reactivation of blood AChE after both 2-PAM and pro-2-PAM treatment confirms that both oximes are active in the PNS (Fig. 7).

3.4 Histopathology of brain sections. Using microscopy, distinct differences between 2-PAM or pro-2-PAM treatments were noted in "A" and "B" sections at the cellular level magnification of 40x, where "A" surveys the piriform cortical neuron layer and "B" the hippocampal pyramidal neuron layer. Under H&E stain, as shown in Fig. 8b and d, which represents a section "A" slice of the brain of DFP or DFP then 2-PAM treated guinea pigs, respectively, the neurons in the piriform cortex showed markedly swollen morphology along with deformed nuclei. These observations contrast with the brain slice from control (PB + atropine, only) and DFP then pro-2-PAM treated animals (Fig. 8 a and c, respectively), where the piriform cortex neurons lack the signs of neuronal necrosis or degeneration seen with 2-PAM treated animals (Fig 8. d).

After fluoro-jade stain, for section "B" slices (Fig. 9), the lower-outside pyramidal layer of the hippocampus exhibited heavily distorted and missing granular cells and neurons in the DFP then 2-PAM treated animals (Fig. 9, b), and dead cells as visualized by the highlighted fluorescent staining were found interspersed and disrupting the order of the pyramidal layer. In contrast, Control (PB + atropine only, Fig. 9, a) hippocampus pyramidal neuron layer was well defined. The DFP then pro-2-PAM layer from treated animals, however, displayed fewer signs of OP toxicity with only swollen cells in this region. Also, the DFP then pro-2-PAM treated animals lacked dead cells as defined by fluoro-jade staining (Fig. 9, c). Slices from control receiving either oxime but no DFP showed no discernable cellular changes in the brain

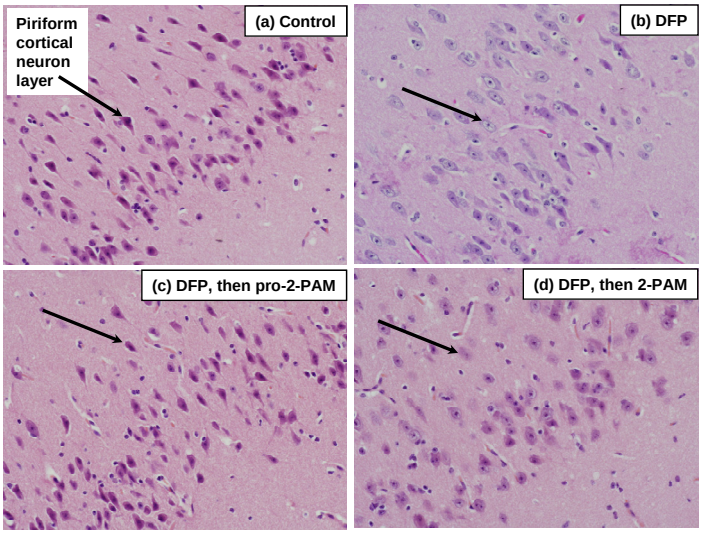

Figure 8. H & E stain of guinea pig brain, 40x magnification of the piriform cortical neuron layer ("A"; see Methods section 2.5). (a) Control brain; (b) brain from animal receiving DFP only, (c) brain from animal receiving DFP followed by the oxime pro-2-PAM, (d) brain from animal receiving DFP followed by the oxime 2-PAM. Black arrow points to piriform neurons.

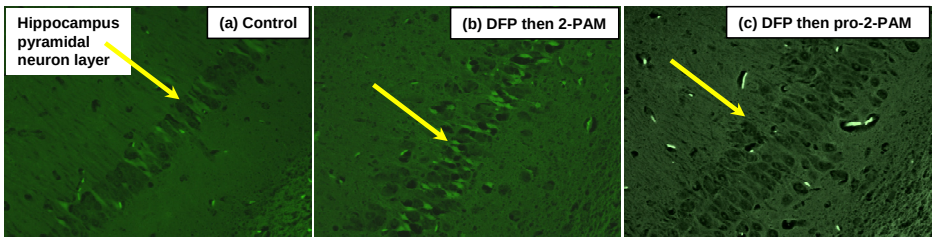

Fig. 9. Fluoro-jade stain of guinea pig brain, 40x magnification, of the hippocampus pyramidal neuron layer ("B); see Methods section 2.5. (a) Control brain; (b) brain from animal receiving DFP followed by the oxime 2-PAM; (c) brain from animal receiving DFP then the oxime pro-2-PAM. Yellow arrow points to hippocampus pyramidal neurons.

regions examined for sections "A" and "B" (histopathology images not shown).

4. CONCLUSIONS

Our hypothesis evolved from the following observations: (a) CNS perturbations, neuronal damage, and long-term sequelae are major components of pesticide and OP toxicity; (b) The resulting neuronal cholinergic crisis leads to over-stimulation of the excitatory amino acid pathway and untreated chemical agent-exposed animals ultimately progress to *status epilepticus*, a life threatening emergency; (c) Oximes reactivate peripherally-inhibited AChEs in the blood and diaphragm; (d) Currently-used oximes do not reactivate CNS AChE because their positively charged chemical structure precludes BBB penetration and the CNS AChE remains inhibited. Based on these observations, our ongoing goal is to develop a non-invasive post-exposure reactivation treatment for pesticide- and OP-induced CNS (and PNS) inhibited AChE.

Chemical warfare agents, including pesticide and OP nerve agents, inhibit ChEs by phosphorylating an active site serine. Oximes have been shown to be effective in reactivating ChEs by nucleophilic attack on the phosphorus atom, resulting in binding of the phosphorus moiety to the oxime with subsequent hydrolysis of the phosphorus-serine bond (Taylor, 1990). The present oxime of choice in the United States is 2-PAM (pralidoxime chloride). Current treatment for acute OP poisoning includes combined administration of 2-PAM (ChE reactivator), atropine (muscarinic receptor antagonist) and diazepam (anticonvulsant) from auto-injectors carried by each soldier (Marrs, et al, 2006). The anticonvulsant is necessary in the current treatment regimen because 2-PAM does not penetrate the BBB. This notable problem means that brain ChEs are not reactivated. The lack of reactivation of CNS AChE most likely led to the long-term post-exposure effects of sarin on the nervous system in victims of the Tokyo Subway Sarin Attack, where chronic decline of psychomotor and memory function still exist more than 12 years after the original sarin exposure (Miyaki et al, 2005, Yamasue et al, 2007).

Especially important for successful long-term recovery from OP exposure is controlling convulsive behavior that can lead to *status epilepticus* and neuropathogenesis; this is the principal reason for developing a CNS penetrating oxime. The guinea pig brains in this study were processed for histopathology from 2 mm coronal sections, and stained with either H&E or fluoro-jade to provide evidence for protection against CNS pathological damage due to the centrally acting OP, DFP. Based on previous studies (Carpentier et al, 2000), damaged neurons demonstrated by H&E are expected to exhibit hypereosinophilic cytoplasm and hyperchromatic nuclei, or less frequently to have pyknotic nuclei. OP-induced neuropathology of brain areas has also been assessed by fluoro-jade staining (Myhrer et al, 2005), and Figures 8 and 9 clearly demonstrate the protection of piriform cortical and hippocampal neurons, respectively, by pro-2-PAM using these techniques.

We have demonstrated that pro-2-PAM restored both CNS AChE activity (Figs. 5 and 6), centrally controlled body temperature (Fig. 4), and eliminated *status epilepticus* originating in the CNS (Fig. 3) even after therapeutic delay of 15 min of *SE*. However, it should be noted that in this study the standard military exposure paradigm was used and only the oxime delivery time was modified. For any CWNA that ages AChE at more than 1 h, such as sarin, the paradigm with later treatments ($\geq$ 15 min demonstrated in this study) will likely be protective. This is not the case for soman, which ages AChE in 2 minutes (Clement, 1982) and will require the 1 min post-exposure treatment with pro-2-PAM for efficacy both in the PNS and CNS. In addition to CNS AChE activation, pro-2-PAM retained its reactivating efficacy in the PNS since AChE in the blood exhibited similar activity with either pro-2-PAM or 2-PAM therapy (Fig. 7). Furthermore, to facilitate submission for regulatory approval and human clinical use, pro-2-PAM is based on and is oxidized *in vivo* in the PNS and CNS to the current FDA approved oxime therapeutic 2-PAM.

In conclusion, given the potential increase in urban terrorism that may include the use of chemical warfare OP agents, Federal, State, and local authorities need a readily available, easily administered, fast acting, PNS and CNS reactivating oxime. The oxime pro-2-PAM fulfills these requirements: our results show that pro-2-PAM crosses the BBB, is rapidly converted inside the brain to the bio-active oxime 2-PAM, and protects against OP-induced seizures through restoration of brain AChE activity. The outcome of this work will provide a non-invasive means of administrating CNS therapeutics for the deleterious effects of OP poisoning, and is directly relevant to use by both soldiers and civilian emergency responders.

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ABBREVIATIONS: ACh, acetylcholine; AChE, acetylcholinesterase; BBB, blood brain barrier; BChE, butyrylcholinesterase; ChE, cholinesterase(s); CNS, central nervous system; CWNA, chemical warfare nerve agent; DFP, diisopropylfluorophosphate; ECG, electrocardiography; EEG, electroencephalography; GA, tabun; GB, sarin; GD, soman; logP, log octanol-water partition coefficient; PB, pyridostigmine bromide, PNS, peripheral nervous system; OP, organophosphate, *SE, status epilepticus*.